

## NEW AFFINITY LIGAND

### Technical field

The present invention relates to a novel IgG-binding compound useful as a ligand for human IgGs of  $\kappa$ -type and functional derivatives thereof. The invention also relates to a separation matrix for use in affinity chromatography comprising said compound and various uses thereof.

### Background

Antibodies, also denoted immunoglobulins, are normally synthesised by lymphoid cells derived from B-lymphocytes of bone marrow. Lymphocytes derived from the same clone produce immunoglobulin of a single amino acid sequence. Lymphocytes cannot be directly cultured over long periods of time to produce substantial amounts of their specific antibody. However, a process of somatic cell fusion, specifically between a lymphocyte and a myeloma cell, has been shown to yield hybrid cells that grow in culture and produce a specific antibody known as a monoclonal antibody. The resulting hybrid cell is known as a hybridoma. A monoclonal antibody belongs to a group of antibodies whose population is substantially homogeneous, i.e. the individual molecules of the antibody population are identical except for naturally occurring mutations.

The development of monoclonal antibody technology has provided an enormous opportunity for science and medicine in implementing research, diagnosis and therapy. Monoclonal antibodies are e.g. used in radioimmunoassays, enzyme-linked immunosorbent assays, immunocytopathology, and flow cytometry for *in vitro* diagnosis, and *in vivo* for diagnosis and immunotherapy of human disease.

Antibodies are grouped into five different types, namely immunoglobulin G (IgG), which is the most prevalent; immunoglobulin A (IgA); immunoglobulin M (IgM); immunoglobulin D (IgD); and immunoglobulin E (IgE). At present,

about thirty percent of the biotechnology-derived drugs under development are based on monoclonal antibodies of type G.

The Y-shaped disposition of the structure of the IgG molecule is well known from standard biochemistry textbooks. In brief, regarding its tertiary structure, one intact IgG molecule consists of six globular regions, each of which is formed by two domains. Regarding its primary structure, an IgG consists of two light chains and two heavy chains, which are covalently linked by disulphide bridges. The two globular parts that correspond to the "base of the Y" form the Fc fragment and are formed by domains consisting of only heavy chain residues. Contrary to this, each of the "arms of the Y" constitutes a Fab fragment with two globular parts each. Each of the globular parts in a Fab fragment is formed when one domain from the light chain contacts one domain from the heavy chain. It is well known that the globular part located further away from the centre of the antibody comprises the regions known as the hypervariable regions as well as the antigen-binding site.

By sequence homology, heavy chains of IgGs can be classified into the four types 1, 2, 3 and 4 whereas light chains fall into two types called  $\lambda$  and  $\kappa$ . In humans, about 40% of the IgG molecules carry a light chain of  $\lambda$  type whereas about 60% carry a light chain of  $\kappa$  type. IgGs built up of both light and heavy chains inherit both types of partitionings. Accordingly, one partitioning divides IgGs into four subclasses IgG1, IgG2, IgG3 and IgG4 as compared to the second partitioning which divides IgGs into two subtypes  $\lambda$  and  $\kappa$ . The same type of classification can be applied to antibody fragments like Fab fragments and so called F(ab')<sub>2</sub> fragments, which consist of two Fab fragments connected by a disulphide.

These days, IgGs are generated according to standard techniques in large quantities in cellular expression systems. The most widely used production method includes purification via chromatography, which due to its versatility and sen-

sitivity to the compounds often is the preferred purification method in the context of biomolecules. The term chromatography embraces a family of closely related separation methods, which are all based on the principle that two mutually immiscible phases are brought into contact. More specifically, the target compound is introduced into a mobile phase, which is contacted with a stationary phase. The target compound will then undergo a series of interactions between the stationary and mobile phases as it is being carried through the system by the mobile phase. The interactions exploit differences in the physical or chemical properties of the components in the sample. The interactions can be based on one or more different principles, such as charge, hydrophobicity, affinity etc. In the context of antibodies, affinity chromatography is the most widely utilised purification scheme. More specifically, affinity chromatography is a highly specific mode of chromatography wherein molecular recognition process takes place between a biospecific ligand and a target substance by a principle of lock-key recognition, which is similar to the enzyme binding to a receptor. For a general review of the principles of affinity chromatography, see e.g. Wilchek, M., and Chaiken, I. 2000. An overview of affinity chromatography. *Methods Mol. Biol.* **147**: 1-6.

Lawrence et al (J.F. Lawrence, C. Ménard, M-C Hennion, V. Pichon, F. Le Goffic, N. Durand in *Journal of Chromatography A*, 732 (1996) 277-281: Use of immunoaffinity chromatography as a simplified cleanup technique for the liquid chromatographic determination of phenylurea herbicides in plant material) describes an evaluation of polyclonal antibodies for cleanup of extracts of food samples. More specifically, antibodies were generated in rabbit after inoculations with an antigen prepared from an urea herbicide. Thus, the antibodies were highly specific to the urea herbicide, which is consequently not useful in any method of general antibody purification.

Another application of urea compounds is provided in EP 0 743 067 (Toray Industries), wherein the compounds are presented as highly selective adsorbing

materials used for elimination or detoxification of superantigens from body fluids. The superantigens described are enterotoxins and exotoxins, which are large proteins.

In the field of affinity chromatography, various patents and patent applications relate to protein A, which is an IgG-binding cell wall protein of the bacteria *Staphylococcus aureus*, and its use as a ligand. For example, PCT/SE83/00297 (Pharmacia Biotech AB) discloses a recombinant form of protein A, wherein a cysteine residue has been added to the protein A molecule to improve its coupling to a separation matrix for subsequent use as an affinity ligand. Further, US patent no. 6,197,927 (assigned to Genentech Inc.) discloses Z domain variants of *Staphylococcal* protein A exhibiting an IgG-binding capacity equivalent to the wild type Z domain, but a significantly reduced size. However, the binding properties of protein A are not ideal. As is well known, protein A binds to IgG molecules from various mammals, with the highest affinity to the human subclasses of IgG1, IgG2 and IgG4. It binds primarily to a surface formed at the juncture of both the second and the third constant domains, known as CH2 and CH3, of IgG located on the Fc fragment. Consequently, protein A cannot be used in affinity purification of any other fragments of IgG than Fc-containing fragments. In addition, even though protein A binds to some Fab fragments, this binding is not generic, since it targets the variable region. However, the interest in Fab and F(ab')<sub>2</sub> fragments has increased lately, since they are smaller than intact IgG molecules but still contain the functional antigen-binding region. Accordingly, the above-mentioned lack of generality becomes another drawback with protein A ligands. Moreover, in attempts to purify IgGs of subclass 3 with protein A-ligands, problems have been reported due to a precipitation of the IgG3 which precipitation is irreversible, thereby causing a loss of purified antibody. Furthermore, protein A exhibits some further drawbacks related to its being a protein. Like most proteins, it is amenable to proteolytic degradation, which may pose serious problems e.g. if a cell lysate is directly applied to a column comprising protein A-based ligand, since most cell lysates

will also comprise various proteases. Further, protein A-based ligands are usually labile to the conventionally used cleaning in place (cip) procedures at high pH conditions, which renders reuse of the column more difficult. In addition, protein A-based affinity ligands have also been known to be unstable under acidic conditions, which may result in an undesired leakage of the ligand during the purification process which will both contaminate the product and impair the quality of the purification system.

Another ligand suggested for use in affinity chromatography has been disclosed in US patent no. 4,977,247, namely the cell wall protein known as protein G. More specifically, protein G exhibits a different affinity to IgGs as compared to protein A. Protein G binds to a highly conserved region of the constant part of the Fab fragment, primarily to residues from the heavy chain, and consequently it has potential to be used as a generic Fab binder. However, it has been reported that protein G has a reduced binding to Fab fragments of type IgG2. In addition, protein G shares most of the disadvantages of protein-based affinity ligands discussed above in relation to protein A. Furthermore, many of the known protein-based affinity ligands have proven to be relatively expensive to produce.

Consequently, there is a need of novel IgG-binding ligands of a more advantageous nature, which are also more cost-effective to produce. Such new ligands should avoid the above-discussed drawbacks, and preferably also involve more preferable binding properties than the hitherto suggested ligands.

In a recent work by the present inventors, which at the time of filing of the present patent application was still not published, a novel binding site that exhibits the spatial conformation of a pocket was identified. The binding pocket was shown to be specific for human kappa IgGs of all subtypes.

The recently identified binding pocket directed the present inventors to a new target on the human IgG molecule in their efforts to find a new affinity ligand with improved properties as compared to the prior art.

#### Summary of the present invention

One object of the present invention is to provide a novel ligand to human IgG-molecules of  $\kappa$ -type, which avoids one or more of the above-discussed disadvantages.

A specific object of the present invention is to provide a novel ligand to human IgG-molecules of  $\kappa$ -type, which is general for all subclasses of said IgGs.

Another object of the invention is to provide a novel ligand to human IgG-molecules of  $\kappa$ -type, which is capable of specific binding to said IgGs.

Yet another object of the present invention is to provide a novel ligand to human IgG-molecules of  $\kappa$ -type, which conforms spatially with a binding pocket defined by the amino acids of the interacting surfaces defined in Fig 2, or with essential parts thereof.

An additional object of the present invention is to provide a novel ligand to human IgG-molecules of  $\kappa$ -type, which exhibits more advantageous chemical properties than protein-based affinity ligands e.g. at extreme pH values and which is more cost-effective to produce.

Further objects and advantages of the present invention will appear from the detailed description that follow below.

#### Brief description of the drawings

Figure 1 shows the executed synthetic route to variations of the substitution pattern of a compound according to the invention and also outlines how in the experimental part below, the compounds in the directed library were provided with a handle for immobilisation.

Figure 2 shows orthographic views of the herein-discussed binding pocket in chicken net model.

Figure 3 shows a selection of compounds according to the invention, wherein the substitution pattern has been varied.

Figure 4 shows orthographic views of some of the compounds derived from AB\_0001250.

Figure 5 A-E show orthographic views of the docked compounds AB\_000125[1-5].

Figure 6A and B show the structure coordinates of the amino acids that form the interacting surfaces of a binding pocket, which is specific for human IgGs of  $\kappa$ -type. Said binding pocket, and compounds comprising said interacting surfaces, were identified by the present inventors and claimed in a separate patent application, which was still pending, but not public at the time of the present filing.

Figure 7 shows the results of affinity chromatography on a separation matrix according to the invention, wherein a Fab-fragment of  $\kappa$ -type is successfully isolated.

Figure 8 shows the results of affinity chromatography on a separation matrix according to the invention, wherein another Fab-fragment of  $\kappa$ -type is successfully isolated.

Figure 9 shows as a comparative test an attempt to isolate a Fab-fragment of lambda-type by affinity chromatography on a separation matrix according to the invention.

### Definitions

The terms "antibody of  $\kappa$  type", "Fab fragment of  $\kappa$  type" and " $F(ab')_2$  fragment of  $\kappa$  type" mean herein an antibody, a Fab fragment and an  $F(ab')_2$  fragment respectively, wherein the light chain is of  $\kappa$  type.

The term "ligand" means herein a chemical entity capable of specific binding to a target.

The term "associating with" refers to a condition of proximity between a chemical entity, or portions thereof, and a target i.e. a binding pocket or binding site on a protein. The association may be non-covalent, wherein the juxta-

position is energetically favoured by hydrogen bonding or van der Waals or electrostatic interactions, or alternatively it may be covalent.

The term "functional derivative" is used to mean a chemical substance that is related structurally and functionally to another substance. Thus, a functional derivative comprises a modified structure from the other substance, and maintains the function of the other substance, which in this instance means that it maintains the ability to interact with the same ligands. Thus, a "functional derivative" can be either a natural variation or fragment thereof, or a recombinantly produced entity. In addition, a "functional derivative" can also comprise added molecules or parts, as long as the described function is essentially retained.

The term "binding pocket", as used herein, refers to a region of a molecule or molecular complex, that, as a result of its hollow shape, favourably contributes to the molecule's association with another chemical entity. The term "interacting surface" means herein a surface comprised of residues capable of interacting with a binding molecule or other entity, e.g. by ionic attraction, hydrogen bonds, Van der Waals interaction etc.

The term "strictly conserved" is used herein to mean that after a sequence alignment of all sequences available from an internationally recognised sequence database, the residue type is exactly the same at a specific position for all aligned sequences. An example of such a database is the non-redundant database provided by the National Centre for Biotechnology Information.

The term "structure coordinates" refers to Cartesian coordinates derived from for example mathematical equations related to the patterns obtained on diffraction of a monochromatic beam of X-rays by the atoms (scattering centres) of a protein or protein-ligand complex in crystal form. The diffraction data are used to calculate an electron density map of the repeating unit of the crystal. The electron density maps are then used to establish the positions of the individual atoms of the protein or protein complex.

A "pharmacophore" is defined herein as the assembled atoms or centres in a target molecule, which have critical interactions with a receptor. Some types



commonly used include hydrogen bond donors; hydrogen bond acceptors; positively or negatively charged centres; aromatic ring centres; and hydrophobic centres.

The term "docking" means herein a fitting operation, wherein the ability of a chemical entity to bind or "dock" to a binding site is evaluated.

The term "library" means a collection of molecules or other chemical entities with different chemical structures and/or properties.

The term a "Conolly surface" defines the surface of the volume accessible to a hard spherical probe of a given radius, usually taken as 1.4Å, which is the radius of water in ice form. This surface can be obtained by "rolling the probe" over the atoms of the protein.

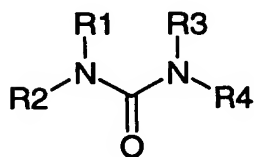
#### Detailed description of the invention

A first aspect of the present invention is a compound capable of associating with human IgGs of  $\kappa$ -type and functional derivatives thereof. More specifically, the present compound is capable of specific and reversible binding to a binding pocket of a human IgG of  $\kappa$ -type, which binding pocket is defined by the structure coordinates for the amino acids that constitute the interacting surfaces as shown in Fig 6. Said binding pocket was recently identified by the present inventors, and is located between the two domains (CH1 and CL) of the constant part of  $\kappa$ -Fab. Thus, the herein discussed binding pocket provides a novel binding site for human IgGs of  $\kappa$ -type, which binding site is a general binding site for all such IgGs as well as fragments or functional derivatives thereof.

The present invention is based on an evaluation of a large number of potential binders to  $\kappa$ -Fab of human IgGs, wherein virtual screening hits were tested with NMR. The results from the NMR was subsequently utilised to derive structure-activity relationships that led to the construction of a pharmacophore, and a library of affinity ligands was then designed to optimise binding and include a handle for immobilisation to a chromatographic support. As will be

disclosed in detail in the Experimental part below, the present inventors have studied different substitution patterns and evaluated a wide range of structures in order to identify the features required for a compound to exhibit a satisfactory binding to human IgGs of  $\kappa$ -type via the above discussed binding pocket.

More specifically, the compound according to the invention is based on an N,N-alkylated urea moiety located between an aromatic part and an aliphatic part. In the most preferred embodiment, the present invention is an IgG-binding compound represented by formula (I) below



(I)

wherein

R<sub>1</sub> is CH<sub>3</sub> or CH<sub>2</sub>CH<sub>3</sub>;

R<sub>2</sub> is a *para* and/or *meta* substituted phenyl group;

R<sub>3</sub> is H, CH<sub>3</sub> or CH<sub>2</sub>CH<sub>3</sub>; and

R<sub>4</sub> is a linear or cyclic aliphatic group, which is optionally substituted, or, wherein

R<sub>1</sub> and R<sub>2</sub> are as stated above while R<sub>3</sub> and R<sub>4</sub> are both parts the same 4- to 6-membered cyclic entity, which is optionally substituted,

and which compound has affinity for human IgG of  $\kappa$ -type.

Thus, in one embodiment, the compound is an affinity ligand with affinity for a Fab fragment of human IgG of  $\kappa$ -type. In some contexts, such an affinity ligand is denoted an affinity adsorbent or an antibody adsorbent.

As the skilled person in this field will easily appreciate, in formula (I), the bonds between the carbonyl carbon and each one of the nitrogen atoms are rotatable. Consequently, position R<sub>1</sub> is equivalent to position R<sub>2</sub> and position R<sub>3</sub>

is equivalent to position  $R_4$ , and the definitions herein will encompass any definition of a compound, wherein  $R_1$  has been interchanged with  $R_2$  as well as when  $R_3$  has been interchanged with  $R_4$ . Likewise, because of the inherent symmetry around the keto group, the pair  $R_1/R_2$  is interchangeable with the pair  $R_3/R_4$  so all these definitions are also included.

In an advantageous embodiment of the compound, in formula (I),  $R_1$  is  $\text{CH}_3$ .

As mentioned above, in formula (I),  $R_2$  is a phenyl group, which may be substituted with one or two halogens, such as F, Cl, Br, or I. Since substituents in *ortho* position have been observed to have a negative impact on binding, any substituents are present in *meta* and/or *para* position. Thus, in a specific embodiment,  $R_2$  is substituted with Cl or F in the *meta* position. In another embodiment,  $R_2$  is substituted with Cl in the *meta* position and F in the *para* position. In another embodiment,  $R_2$  is substituted with F in the *meta* position and Cl in the *para* position. In yet another embodiment,  $R_2$  is substituted with Cl in *meta* and *para* position.

Alternatively, or additionally, the  $R_2$  phenyl group is substituted with one or more oxygen-comprising groups. Thus, in one embodiment,  $R_2$  is a substituted phenyl group and the substituents are selected from the group that consists of F, Cl, Br, I and OH, preferably F and Cl.

In a specific embodiment,  $R_2$  is substituted in the *para* and/or *meta* position with a group defined as  $-\text{O}-R_5$ , wherein  $R_5$  is  $\text{CH}_3$  or  $\text{CH}_2\text{CH}_3$ , and preferably  $\text{CH}_3$ .

As appears from the modelling described in the experimental part below, when the present compound binds to an IgG molecule,  $R_2$  will be located in the inner part of the pocket and hence interact with the inner amino acids of the interacting surfaces of the binding pocket. Larger ring-systems than six-membered

rings were according to NMR screening described in the experimental part below found to have a negative influence on binding, and are hence avoided. Also, as mentioned above, in the most preferred embodiment, the aromatic group does not comprise any heteroatoms, since especially the presence of nitrogen atom(s) in the ring has been observed to have a negative impact on binding. However, in an alternative embodiment, the invention is a compound represented by the chemical formula (I) as defined above, wherein  $R_2$  is another aromatic group than phenyl. In the most preferred embodiment of this alternative,  $R_2$  comprises thiophene.

As mentioned above,  $R_3$  can be H or  $CH_3$  or  $CH_2CH_3$ .

As mentioned above, in formula (I),  $R_4$  can be a linear or cyclic aliphatic group, which is substituted or unsubstituted. In this context, an aliphatic group can be any linear or branched carbon chain interrupted by any heteroatom, as long as the compound fits sufficiently well in the herein-defined binding pocket to provide binding thereof. In one embodiment, the aliphatic chain comprises one or more carbonyl group(s).

In one alternative embodiment,  $R_4$  is an aromatic group that comprises a phenyl group. In one embodiment, said phenyl group is substituted in the *ortho* and/or *meta* and/or *para* position. In a specific embodiment, said phenyl group comprises one or more heteroatoms, such as N, S etc.

In a specific embodiment,  $R_4$  can be a methyl-substituted amino acid residue, or a derivative thereof. Thus, in a specific embodiment,  $R_4$  is selected from the group that consists of aliphatic amino acid residues, hydroxyl-containing amino acid residues, sulphur-containing amino acid residues, aromatic amino acid residues, acidic amino acid residues, basic amino acid residues or imino-containing amino acid residues, or any derivative thereof.

In a specific embodiment, which is especially advantageous if the compound is to be used in a form immobilised to a solid support, e.g. as a ligand in affinity chromatography, the aliphatic group  $R_4$  also comprises terminating functionalities useful for such immobilisation. Thus, in one embodiment, an aliphatic group is a linear or branched carbon chain as discussed above, which is terminated with a carboxylic acid i.e.  $-\text{COOH}$ . In an alternative embodiment, the aliphatic group is terminated with a carboxylic acid derivative, such as an ester, a halide, an amide, a nitrile or the like. In an alternative embodiment, an aliphatic group is a linear or branched carbon chain as discussed above, which is terminated with nitrogen, oxygen, sulphur or any derivative thereof. Such derivatives are well-known to the skilled person in this field, and are also useful for immobilisation. As mentioned above, the only limitation in this context is that the aliphatic group does not impair the binding of the compound to the herein defined binding pocket.

In another embodiment, in formula (I),  $R_4$  is  $\text{CH}_3$ . In a specific embodiment, both  $R_3$  and  $R_4$  are  $\text{CH}_3$ . In a specific embodiment, in formula (I),  $R_1$  is  $\text{CH}_3$ ;  $R_2$  is a phenyl group that has been substituted with Cl in *meta* and *para* position;  $R_3$  is  $\text{CH}_3$ ; and  $R_4$  is  $\text{CH}_3$ . In one embodiment, the present compound is selected from the compounds shown in Figure 3.

As also appears from the above, in an alternative embodiment,  $R_3$  and  $R_4$  are parts of a 4- to 6-membered cyclic entity. In an advantageous embodiment, the cyclic entity is 3- to 5-membered. Consequently, said cyclic entity comprises the N of Formula (I),  $R_3$  and  $R_4$  and optionally 1 or 2 other atoms, which may be carbon atoms or heteroatoms. In the most preferred embodiment, the  $R_3$  and  $R_4$  substituents constitute an amino acid derivative. In one embodiment,  $R_3$  and  $R_4$  are part of a 5-membered cyclic entity, which in turn is substituted, preferably with a group useful for immobilisation as discussed above. In a specific embodiment, a 5-membered cyclic entity is substituted in the position adjacent to the N with a  $\text{C(O)-O-CH}_3$  group, and consequently the  $R_3$  and  $R_4$  substitu-

ents of this embodiment are parts of a D-proline derivative. This specific embodiment is denoted AB\_0003290 in Figure 3.

Furthermore, the present invention also encompasses a compound, which is basically represented by formula (I) above, but wherein  $R_1$  and  $R_3$  are carbon atoms connected to each other to form a cyclic structure. In this embodiment,  $R_3$  is a carbonyl group. In this embodiment,  $R_4$  is preferably a phenyl group. Thus, this embodiment of the compound is known as 1,3-diphenylimidazolidine-2,4-dione.

In order to provide the best binding to the herein-discussed binding pocket of a human IgG of  $\kappa$ -type, or to a functional derivative thereof, it is preferable that the compound has a non-planar geometry. In the context of the binding pocket, it is noted that the present compound is capable of binding to binding pockets not only of the exact defined structure coordinates as defined herein, but also to pockets defined by interacting surfaces having a mean square deviation from the backbone atoms of the disclosed binding pocket amino acids of not more than  $2.0\text{\AA}$ . In a preferred embodiment, said deviation is not more than about  $1.5\text{\AA}$  and in the most preferred embodiment, said deviation is not more than  $1.0\text{\AA}$ . In one embodiment, the present compound is capable of binding to a human IgG or a functional derivative thereof with a binding constant of at least  $10^{-3}\text{ M}$ , preferably at least  $10^{-6}\text{ M}$  and most preferably at least  $10^{-8}\text{ M}$ . Thus, illustrative intervals of such binding are e.g.  $10^{-3}\text{ M}^{-4}$  to  $10^{-8}\text{ M}$ , such as  $10^{-3}\text{ M}^{-4}$  to  $10^{-6}\text{ M}$  or  $10^{-6}$  to  $10^{-8}\text{ M}$ .

In a specific embodiment, the present compound is capable of binding to a human IgG of  $\kappa$ -type, or a functional derivative thereof, via a binding pocket formed between two polypeptides, wherein the first polypeptide is the portion of a human IgG  $\kappa$  light chain that starts at one of amino acids 93 to 110 and ends at one of amino acids 187 to 214 of human IgG  $\kappa$  light chain and the second polypeptide is the portion of a human IgG heavy chain that starts at one of

amino acids 106 to 128 and ends at one of amino acids 215 to 225 of human IgG heavy chain. In the herein used enumeration of amino acids refers to a human sequence wherein no. 93 is the first amino acid of the constant domain, as also used in Figure 6.

Thus, the IgG-binding compounds according to the present invention are in general smaller than the prior art affinity ligands used for antibody isolation. In addition, the compounds according to the invention are organic molecules that lack the peptide structure of e.g. protein A- and protein G-based ligands, which in general renders them less susceptible to extreme pH values. Naturally, they are not as susceptible to proteolytic degradation, or any other kind of degradation, as the protein-based prior art ligands either. In addition, the present compounds are more cost-effective to produce.

The compound according to the invention can be prepared by the skilled person in this field using well-known methods, as illustrated e.g. in Figure 1 below and as explained in the experimental part below under "Synthesis".

A second aspect of the invention is the use of a compound as defined above for selective binding of human IgG of  $\kappa$ -type, or a functional derivative thereof. In the present context, it is understood that the encompassed derivatives can be any human  $\kappa$ -Fab constant part- comprising compounds, i.e. any composition comprising the globular region of an IgG molecule formed by the first constant domain of the heavy chain (CH1) and the constant domain of the light chain (CL). Thus the term includes any of the following terms which are well known from standard IgG terminology: Intact IgG molecules,  $F(ab')_2$  fragments, Fab' fragments, Fab fragments and by definition the globular region named itself, all of which have human sequences and light chains of  $\kappa$ -type. This definition includes also any modifications of named IgG or named antibody fragments including even chimeric molecules formed in one part of one of said compositions and in another part of any of the following proteins, peptides, carbohy-

drates, lipids or any other organic or inorganic entity and chimeric combinations thereof and also any of the above-mentioned covalently attached to solid phase.

The present invention also encompasses a sorption complex comprised of a compound as defined above directly linked to the Fab fragment of a human IgG of  $\kappa$ -type, or a functional derivative thereof. More specifically, the compound is linked to the Fab fragment of said antibody, and more specifically to the herein described binding pocket. Such a sorption complex will form as the compound according to the invention is contacted with a solution comprising human IgG's of  $\kappa$ -type, or a functional derivative thereof, under suitable conditions. The skilled person in this field can easily select such conditions and adjust pH, ionic strength etc to provide or to break up the complex.

Another aspect of the invention is a separation matrix for use in affinity chromatography, wherein the ligands comprises at least one compound as defined above. In a specific embodiment, the ligands have been coupled to a support via linkers. The present matrix can e.g. be in the form of separate particles, preferably porous and essentially spherical particles; a monolith; or a membrane.

The present invention also encompasses a system suitable for affinity chromatography, which is comprised of a separation matrix as defined above packed in a column. The column may be of a size suitable for analytical scale or for large scale chromatography.

Suitable support materials are well known. In one embodiment, the support is a natural polymer, such as agarose, alginate, carrageenan, gelatine etc. Such natural polymers are known to form physically cross-linked networks spontaneously on cooling or on addition of divalent metal ions, and chemical cross-linkers can be added if desired. This kind of supports is easily prepared ac-



according to standard methods, such as inverse suspension gelation (S Hjertén: *Biochim Biophys Acta* 79(2), 393-398 (1964)). In another embodiment, the support is comprised of cross-linked synthetic polymers, such as styrene or styrene derivatives, divinylbenzene, acrylamides, acrylate esters, methacrylate esters, vinyl esters, vinyl amides etc. Such polymers are also easily produced according to standard methods, see e.g. "Styrene based polymer supports developed by suspension polymerization" (R Arshady: *Chimica e L'Industria* 70(9), 70-75 (1988)). Thus, in summary, the support material can in principle be any material that allows the covalent coupling of the IgG-binding compound discussed above, such as the above-discussed polymers, inorganic materials, such as silica, ceramics etc.

Many well-known methods are available for immobilising ligands to a support through suitable functional groups. As the skilled person in this field will realise, the exact choice of coupling method will depend on the structure of the ligand to be immobilised. In one embodiment, the support has hydrophilic surfaces, and if porous, the surfaces of the pores are also hydrophilic. This is advantageous in order to avoid or at least reduce any non-specific protein interactions. It is also advantageous if the surfaces have a high density of groups available for coupling of ligands. Such coupling groups are commonly hydroxyl groups, but may also be allyl groups i.e. double bonds available for grafting, amines, thiols, epoxides and the like. If the support material has undesirable surface properties, it is possible to coat it with a hydrophilic polyhydroxy-functional material before coupling the ligand. The techniques and considerations for coupling of affinity ligands to a suitable support to prepare a separation matrix are well known in this field, see e.g. WO 98/33572 for a detailed review of coupling chemistry as well as suitable linking molecules, therein denoted "extenders".

Another aspect of the invention is a generic method of isolating or separating a target compound, i.e. a human IgG of  $\kappa$ -type, or a functional derivative thereof,

from other components in a liquid, wherein a compound or a separation matrix as defined above is used. In the context of immunology, the separation matrix is often denoted an "immunsorbent". In the most preferred embodiment, the present method is affinity chromatography, which is a widely used and well-known separation technique. In brief, in a first step, a solution comprising the desired antibodies is passed over a separation matrix under conditions allowing adsorption of the antibody to ligands present on said matrix. Such conditions are controlled e.g. by pH and/or salt concentration i.e. ionic strength in the solution. Care should be taken not to exceed the capacity of the matrix, i.e. the flow should be sufficiently slow to allow a satisfactory adsorption. In this step, other components of the solution will pass through in principle unimpeded. Optionally, the matrix is then washed, e.g. with an aqueous solution, in order to remove retained and/or loosely bound substances. In a next step, a second solution denoted an eluent is passed over the matrix under conditions that provide desorption i.e. release of the desired antibody. Such conditions are commonly provided by a change of the pH, the salt concentration i.e. ionic strength, hydrophobicity etc. Various elution schemes are known, such as gradient elution and step-wise elution. Elution can also be provided by a second solution comprising a competitive substance, which will replace the desired antibody on the matrix.

In an alternative embodiment, the compound according to the invention is used in site-specific modification of a human IgG of  $\kappa$ -type, or a functional derivative thereof. More specifically, a human IgG of  $\kappa$ -type, or a functional derivative thereof, can be modified by binding a compound as defined above selectively to the binding pocket identified by the present inventors. In a specific embodiment, the modification is a stabilisation of Fab-folding.

In an alternative embodiment, the present compound is used in an immunological assay for detection of a human IgG of  $\kappa$ -type, or a functional derivative thereof. In this case, the compound is preferably labelled with a suitable detect-

able label as conventionally used, such as a fluorescent label, a luminescent label, a chemiluminescent label, an enzyme label, a radioactive label, an absorbance label etc. Such assays may be in solution or on solid phase. In one embodiment, the human  $\kappa$ -Fab constant part-comprising composition is a human IgG or a fragment thereof. In the preferred embodiment, the present assay is a competitive assay, wherein the ability of a candidate ligand to displace a known ligand's binding to a compound or binding pocket as defined above is evaluated.

#### Detailed description of the drawings

Figure 1 shows the executed synthetic route to i) variations of the substitution pattern and ii) provide the compounds in the directed library with a handle for immobilisation as discussed below in Example 2. To the top-left, the original hit AB\_0001250 is shown. The synthesis will be described in detail below in the section Materials and methods.

Figure 2 shows orthographic views of the herein-discussed binding pocket in chicken net model. The amino acid residues forming the pocket are shown in stick model and the corresponding structure coordinates are presented in Figure 6. Docked hit AB\_0001250 is shown in space-fill model to illustrate the possibilities of the pocket to harbour a substituted phenyl ring.

Figure 3 shows a selection of compounds according to the invention, wherein the substitution pattern of  $R_1$ ,  $R_2$  as well as  $R_3$  and  $R_4$  has been varied. A central N,N-alkylated urea moiety as well as a *para* and/or *meta* substituted phenyl groups are present in all the compounds.

Figure 4 shows orthographic views of compounds derived from AB\_0001250. Five docked hits superimpose very well onto the original hit AB\_0001250.

Figure 5 A-E show orthographic views of the docked compounds AB\_000125[1-5] in the binding pocket, as discussed in more detail in the experimental part below.

Figure 6 A and B show the structure coordinates of the amino acids that form the interacting surfaces of the binding pocket shown in Figure 2, which is spe-

cific for human IgGs of  $\kappa$ -type. Figure 6A shows the coordinates of the light chain, while Figure 6B shows the heavy chain. More specifically, the structure coordinates shown form a small pocket in between the two domains (CH1 and CL) of the constant part of  $\kappa$ -Fab and constitutes a novel target binding site. The residues forming the pocket together with some residues located at the entrance and contributing significantly to the topology of the putative binding site have been identified as follows. From the light chain, there are **Q124, S127, G128, T129, S131, V133, G157, N158, S159, Q160, E161, S162, S176, S177, T178, T180, L181**, and they are all strictly conserved for all sequences of  $\kappa$ -type identified in a sequence homology search. The residues from the heavy chain are **K126, P128, S129, F131, L133, L150, K152, D153, F175, P176, V178, L179, Q180, S181, S182, L184, S186, L187 and S188**, bold being strictly conserved and remaining highly conserved. The structure coordinates of the full amino acid sequence of a human IgG of  $\kappa$ -type can be obtained from the Protein Data Bank, accession code 1vge, e.g. at <http://www.rcsb.org/pdb/>. Figure 7 illustrates how a Fab-fragment of a monoclonal antibody of kappa-type can be isolated by affinity chromatography using a separation matrix according to the invention, as described in Example 6 below. Injection of the monoclonal ABFab-K1 on the AB\_0003291-containing medium according to the invention in PBS, 1 M  $(\text{NH}_4)_2\text{SO}_4$ , pH 7 at 0 ml. Cleaning in place (CIP) starts at 10.0 ml. The small peak at 1.07 ml is due to injection effect. The protein is not totally removed from the column during the CIP. Evidently, the affinity column is able to bind the monoclonal ABFab-K1.

Figure 8 illustrates how another Fab-fragment of a monoclonal antibody of kappa-type can be isolated by affinity chromatography using a separation matrix according to the invention, as described in Example 6 below. Injection of the monoclonal ABFab-K2 on the AB\_0003291-containing media in PBS, 1 M  $(\text{NH}_4)_2\text{SO}_4$ , pH 7 at 0 ml. CIP starts at 10.0 ml. The small peak at 1.09 ml is due to injection effect. The protein is not totally removed from the column during the CIP. Evidently, this affinity column is also able to bind the monoclonal ABFab-K2, and the compound according to the invention can conse-

quently be described as a ligand useful as a general binder of human IgG Fab fragments of  $\kappa$ -type.

Figure 9 illustrates as a comparative test how a Fab-fragment of a monoclonal antibody of lambda-type is tested in affinity chromatography on a separation matrix according to the invention, as described in Example 6 below. Injection of the monoclonal ABFab-L2 on the AB\_0003291-containing media in PBS, 1 M  $(\text{NH}_4)_2\text{SO}_4$ , pH 7 at 0 ml. CIP starts at 10.0 ml. From this figure, it clearly appears that the ABFab-L2 directly comes off the affinity column and is found in the flow-through. Accordingly, the separation matrix according to the invention is not suitable for isolation of Fab-fragments of lambda-type, and confirms the statement above that the compound according to the invention is a binder of human IgG Fab fragments of  $\kappa$ -type, but not of lambda-type.

## EXPERIMENTAL PART

Below, the present invention will be explained in more detail by way of examples, which however are not to be construed as limiting the present invention as defined by the appended claims. All references given below and elsewhere in the present specification are hereby included herein by reference.

### Materials and Methods

#### Molecular Modelling

Compounds of the directed library were sketched with MDL ISIS/draw and transferred to an OCTANE™ (Silicon Graphics Inc.®) workstation provided with two 195MHz R10000 processors. The program package SYBYL® (Tripos Inc., 2000) was used for all remaining modelling.

#### Preparation of compounds for docking

The structures of the compounds were transformed into 3D using the program CONCORD and ionised to reflect their most probable protonation state at pH 7. The coordinates were then subject to 500 cycles of minimisation using the

MMFF94 force field (Halgren 1996 – Halgren, T. 1996. Merck molecular force field. I. Basis, form, scope, parameterisation, and performance of MMFF94. *J. Comp. Chem.* **17**: 490-519. ).

#### Docking of prepared molecules

Docking simulations have been performed with the program FlexX™ (Rarey et al. 1996 Rarey, M., Kramer, B., Lengauer, T., and Klebe G. 1996. A fast flexible docking method using an incremental construction algorithm. *J. Mol. Biol.* **261**: 470-489.) which is part of the SYBYL package. FlexX allows flexibility in the ligands, keeping the receptor fixed. All the relevant receptor information necessary for the docking simulations is stored in the receptor definition file (rd file). FlexX uses formal charges, which were turned on during the docking simulations. The protein structure used was the highest-resolution (2.0 Å) crystal structure of  $\kappa$ -Fab (accession code to the Protein Data Bank 1vge, Chacko *et al.*, 1996 Chacko, S., Padlan, E. A., Portolano, S., McLachlan, S. M., Rapoport, B.: Structural studies of human autoantibodies. Crystal structure of a thyroid peroxidase autoantibody Fab. *J Biol Chem* 271 pp. 12191 (1996)). The following residues were included in the definition of the binding site: from the light chain: Ser-131, Val-133, Ser-159, Gln-160, Glu-161, Ser-162, Ser-176, Thr-178, and Thr-180. From the heavy chain: Leu-150, Lys-152, Phe-175, Pro-176, Val-178, Gln-180, Ser-186, Leu-187, Ser-188. All of these residues have previously been shown by the present inventors to be strictly conserved as observed from a sequence alignment and are a subset of the identified pocket. The subset was created by taking all residues with at least one atom at a distance of at least 4 Å from the docked hit AB\_0001250 and subsequently by including some additional residues to complete a Connolly surface of the pocket surrounding the docked hit. In the protein structure, the  $\epsilon$  carbonyl oxygen of H:Gln-180 is located 2.5Å away from one of the  $\delta$  carboxyl oxygens of H:Asp153. This was assumed to be an error due to misinterpretation of the electron density of the carboxamide terminal group of H:Gln-180, and the group was consequently flipped around 180°. In this corrected structure, the  $\epsilon$

nitrogen of Gln-180 from the heavy chain is at favourable hydrogen bonding distance to the carboxyl oxygen of H:Asp153. Otherwise, defaults have been used when creating the rd file and no special customisations have been done. When necessary the *SYBYL LINE NOTATION (sln) core* option of FlexX in SYBYL was applied to bias the docking towards conformations that were compatible with the expected binding mode with the phenyl ring inside the pocket. The *sln core* option was applied with input N(C(NCH3)=O)(C[9]:CH:CH:C:C:CH:@9)CH3 to indicate to the program to start fragment build-up using a common substructure of the six compounds in the directed library. Prior to docking, all water molecules were removed. The 30 best ranked conformations and their FlexX score were saved for each molecule.

#### Synthesis of library based on AB\_0001250

##### Synthesis of 4-(methylamino) butyric acid methyl ester

4-(methylamino) butyric acid·HCl was dissolved in methanol and thionyl chloride in catalytic amount was added drop by drop. The reaction mixture was stirred at 0 °C for 30 min. Thereafter, the solvent was reduced *in vacu*, yielding a white solid.

##### Synthesis of 3,4-dichloro-/(N-methyl)-aniline

3,4-dichloro aniline (40 mmol, 5 g) was dissolved in 400 mL of DCM. To this solution was added iodo methane (40 mL), triethyl amine (5 mL), and NaH (40 mmol, 3.8 g). The resulting mixture was stirred at ambient temperature over night, where after small aliquots of water summing up to a total of 50 mL of water was added, followed of an additional hour of stirring. The reaction mixture was transferred to a separation funnel and extracted with 5 % sodium thio-sulphate, dried over magnesium sulphate and concentrated *in vacu* to almost complete dryness. The material was separated by silica chromatography (pentane:ether – 8:2), the appropriate fractions were collected and concentrated *in vacu* to almost complete dryness, yielding 3g of material including some sol-

vent. The correct material was indicated by LC-MS analysis. This material was directly used in the subsequent step.

#### General method for synthesis of N-methylated aniline derivatives

The aniline derivative was dissolved in DCM and sodium hydride (in the case of AB\_0001253 sodium bis(trimethylsilyl) amide) (1.5 eq) and di-tertbutyl-dicarbonate (1.3 eq) was added followed by stirring at room temperature over night. The reaction mixture was transferred to a separatory funnel and extracted with water, dried over magnesium sulphate, and concentrated *in vacu*. The crude product was dissolved in THF and lithium alumina hydride (1.2 – 2 eq) was added and the reaction mixture was refluxed until completion as indicated by LC-MS. Thereafter the mixture was filtered. This filtrate was used directly in the subsequent step.

#### General method for synthesis of urea derivatives

To a THF solution of the N-methylated aniline (or the non-N-methylated aniline derivative) was added phosgene (20% in toluene) in large excess and the reaction mixture was stirred at room temperature for 30 min, concentrated *in vacu*, and re-dissolved in DCM. To this solution was added an excess of triethyl amine and 4-(methylamino) butyric acid methyl ester (or 4-amino butyric acid methyl ester) (approx. 1 eq). The reaction mixture was stirred at room temperature for 3 hours, concentrated *in vacu*, and purified by RP-HPLC.

#### General method for hydrolysis of methylesters

The methyl ester of the urea derivative (0.5 g) was dissolved in methanol (10 mL) and lithium hydroxide (0.25 g) was added. The resulting mixture was stirred at ambient temperature for 5 hours, neutralised with 1 M HCl, and concentrated *in vacu*. The resulting material was purified by RP-HPLC.

#### Synthesis of 1-(3,4-dichlorophenyl)-1,3-dimethyl-3-butyric acid urea

3,4-dichloro-N-methyl-aniline (all material from previous description) was dissolved in 200 mL of DCM. To the solution was added phosgene ( 20 mL, 20%



sol. in toluene) and the mixture was stirred for 30 minutes at ambient temperature. The solvent was removed *in vacu* and an additional 100 mL of DCM was added, followed by removal of the added solvent *in vacu*.

The remaining solid was dissolved in 200 mL of DCM and 4-methyl-4-amino butyric acid (2 g) was added followed by the addition of triethyl amine (5 mL). The resulting mixture was stirred at ambient temperature during 2 hours. Thereafter, the reaction mixture was transferred to a separation funnel and partitioned between DCM and water. The organic phase was isolated, dried over magnesium sulphate, and concentrated *in vacu*. The remaining material was purified by silica column chromatography (DCM:Et-OH – 9:1), the appropriate fractions were collected and concentrated *in vacu* to yield 1.4 g of the desired material as a clear oil.

#### Example 1: Binding test using NMR

All NMR experiments were performed at 298 K on a Bruker Avance 500 MHz spectrometer. The 1D saturation transfer difference method (STD-NMR) was used as screening assay (Mayer M. and Meyer B. 1999. Characterisation of Ligand Binding by Saturation Transfer Difference NMR Spectroscopy. *Angew. Chem., Int. Ed.* **38**: 1784-1788). The resulting STD-NMR spectrum shows the difference between spectra recorded with on- and off-resonance irradiation of the protein, respectively. The two spectra are recorded in the same experiment in an interleaved fashion. If the resulting STD-NMR spectrum shows the same signals as the reference  $^1\text{H}$ -NMR spectrum of the ligand the result is regarded as positive *i.e.* the ligand must have contacted the protein. Ligands that do not have any contact with the protein or are very tightly bound to the protein will not give any signal in the resulting STD-NMR spectrum. It has been shown that the method is capable of detecting ligands with dissociation constants between  $10^{-3}$  and  $10^{-8}$  M (Mayer M. and Meyer B. 1999. Characterisation of Ligand Binding by Saturation Transfer Difference NMR Spectroscopy. *Angew. Chem., Int. Ed.* **38**: 1784-1788). The strength of the STD-NMR signal depends upon

several factors including protein size, offset and duration of the on-resonance irradiation, the dissociation rate constant and the excess of ligand. The STD-NMR method is advantageous in that the detection limits can be tuned for binding by varying the protein concentration while keeping the ligand concentration constant. Under such conditions, at higher protein concentrations the weak to medium binders are detected, whereas at lower protein concentrations only medium binders are detected. For instance, it has been shown before for another enzyme system that both  $\mu\text{M}$  and  $\text{mM}$  binders were detected at a protein concentration of  $35\ \mu\text{M}$  whereas only  $\mu\text{M}$  binders were detected at protein concentrations of  $1\ \mu\text{M}$  and  $100\ \text{nM}$  (Peng J. W., Lepre C. A., Fejzo J., Abdul-Manan N. and Moore J. M. 2001, Nuclear Magnetic Resonance-Based Approaches for Lead Generation in Drug Discovery, *Methods in Enzymology*. **338**: 202-230). It should be noted that the signal intensity at one specific protein concentration should not be taken as a direct measure of the binding strength. For instance, in the same study, a  $\text{mM}$  binder showed a stronger signal as compared to a  $\mu\text{M}$  binder at  $35\ \mu\text{M}$  protein concentration whereas when the protein concentration was reduced to  $1\ \mu\text{M}$  the signal from the weaker binder vanished. On the other hand the signal of the  $\mu\text{M}$  binder became even stronger than before (Peng J. W., Lepre C. A., Fejzo J., Abdul-Manan N. and Moore J. M. 2001, Nuclear Magnetic Resonance-Based Approaches for Lead Generation in Drug Discovery, *Methods in Enzymology*. **338**: 202-230).

Here, three different antibody concentrations were used, namely,  $0.5\ \mu\text{M}$ ,  $100\ \text{nM}$  and  $20\ \text{nM}$ . The antibody used was a human Fab of  $\kappa$ -type. In all cases ligands were tested one-by-one. On-resonance irradiation was set at  $0\ \text{ppm}$  and off-resonance irradiation was set at  $-40\ \text{ppm}$ . Irradiation time in each scan was  $2\ \text{s}$  and  $16\text{K}$  data points were collected with  $1024$  scans in total. Compounds for testing were dissolved in  $\text{DMSO}_{d6}$  to a concentration of  $50\ \text{mM}$  and  $5\ \mu\text{L}$  of the concentrated ligand solution was added to  $495\ \mu\text{L}$  buffer solution. The samples thus consisted of  $0.5\ \text{mM}$  ligand,  $20\ \text{mM}$  phosphate buffer,  $100\ \text{mM}$   $\text{NaCl}$  and  $5\%$   $\text{DMSO}_{d6}$  in  $\text{D}_2\text{O}$  at  $\text{pD}\ 7.5$ , uncorrected reading on pH-meter.

Compounds were initially tested for binding with 0.5  $\mu$ M antibody. Interesting ligands were further tested with protein concentrations of 100 or 20 nM. A one-dimensional  $^1\text{H}$ -spectrum was acquired first as reference spectrum and subsequently a saturation transfer difference (STD) spectrum was acquired. Each analysis took 60 minutes on the spectrometer.

The results are shown in Table 1 below, wherein the results from NMR screening are compiled.

Table 1: Results from the NMR screening

Concentration code as follows: conc. 1 means 500, conc 2 100 and conc 3 20 nM protein. NMR signal code: 0 no, 1 weak and 2 strong signal.

ID	Chemical name	conc 1	conc 2	conc 3	ctrl
AB_0000510	alpha-pyridoin	0			
AB_0000530	1-(3-chlorophenyl)-3-methyl-2-pyrazolin-5-one	0			
AB_0000540	1,3-diphenylparabanic acid	0			
AB_0000580	n1-(3-chloro-4-fluorophenyl)-2-[(4,6-dimethylpyrimidin-2-yl)thio]acetamide	1	0		
AB_0000600	3-phenyl-1,2,4-benzotriazine	1	1		0
AB_0000610	2-(4-chlorophenyl)-2,3-dihydro-1h-pyrrolo[3,4-c]pyridine-1,3-dione	0			
AB_0000630	n1-(2,3,4-trifluorophenyl)-2-(1,2,4-oxadiazol-3-yl)acetamide	0			
AB_0000670	methyl n-[(5-methyl-4-phenyl-1,3-oxazol-2-yl)carbonyl]carbamate	2	0		
AB_0000690	n1-(2,4-difluorophenyl)-2-(1,2,4-oxadiazol-3-yl)acetamide	0			
AB_0000700	3,6-di-2-pyridyl-1,2,4,5-tetrazine	0			
AB_0000730	2-benzylidene-1,3-indandione	0			
AB_0000740	5-phenyl-1,2,4-oxadiazol-3-yl n-(4-fluorophenyl)carbamate	0			
AB_0000750	n-(5,5-dimethyl-7-oxo-4,5,6,7-tetrahydro-benzothiazol-2-yl)-nicotinamide	0			
AB_0000760	5-(2-phenyl-1,3-thiazol-4-yl)-	1	0		

AB_0000790	1,3,4-oxadiazol-2-ylhydrosulfide 3-[2-oxo-2-(2-pyridyl)ethyl]-1,3-dihydroisobenzofuran-1-one	0			
AB_0000810	2-phenoxy-2-phenyl-1-ethanol	2	1		0
AB_0000860	1,3-diphenylimidazolidine-2,4-dione	2	2	1	0
AB_0000880	5-bromo-3-phenyl-thiazolidine-2,4-dione	0			
AB_0000900	1-(2-naphthoyl)imidazole	0			
AB_0000910	3-bromo-4-methoxyphenylacetone	2	2	0	0
AB_0000930	3-chloro-1-phenyl-pyrrole-2,5-dione	0			
AB_0000990	3-butyl-2-hydroxy-4h-pyrido[1,2-a]pyrimidin-4-one	0			
AB_0001000	3-(benzylamino)-1,1,1-trifluoro-2-propanol	1	0		
AB_0001010	2-[5-(2-fluorobenzoyl)-2-thienyl]acetonitrile	2	2	2	1
AB_0001020	5-(3,5-difluorobenzyl)-3-(2-thienyl)-1,2,4-oxadiazole	1	0		
AB_0001030	2-chloro-3-(trifluoromethyl)benzaldehyde	1	0		
AB_0001040	2-hydroxy-3-(2-methyl-1-propenyl)-1,4-naphthoquinone	1	1		0
AB_0001060	2-(2-imino-thiazol-3-yl)-1-naphthalen-2-yl-ethanone	2	0		
AB_0001070	imiloxan hydrochloride	1	1		
AB_0001080	2-(benzylthio)-5-methyl-4,5-dihydro-1h-imidazol-3-ium chloride	0			
AB_0001090	n-(3-chlorophenyl)-maleimide	1	0		
AB_0001100	ethyl 4-oxo-1,4-dihydroquinoline-3-carboxylate	0			
AB_0001130	3-amino-n-[2-(methylthio)ethyl]-4-oxo-3,4-dihydroquinazoline-2-carboxamide	0			
AB_0001150	1-[(3,4-dichlorobenzyl)oxy]-1h-imidazole	2	0		
AB_0001170	3-[(2,4-dichlorobenzyl)amino]-1,1,1-trifluoro-2-propanol	0			
AB_0001180	3-oxo-2-phenyl-2,3-dihydro-4-pyridazinecarboxylic acid	0			
AB_0001190	4-[1-(2-phenylethyl)-(1h)-pyrazol-4-yl]pyridine	2	0		
AB_0001200	methyl 1-hydroxy-2-naphthoate	1	0		
AB_0001220	1-(3-trifluoromethyl-phenyl)imidazole	2	0		
AB_0001230	(2-naphthoxy)acetic acid, n-hydroxysuccinimide ester	0			

AB_0001240	methyl 3-(5-chloro-2-methoxyphenyl)-2,3-epoxypropionate	1	1	0
AB_0001250	1-(3,4-dichlorophenyl)-1,3,3-trimethylurea	2	2	1
AB_0001260	2-pyridyl 2-(2,3-dihydro-1,4-benzodioxin-2-yl)-1,3-thiazole-4-carbothioate	0		0
AB_0001270	1-[(4-chlorobenzyl)amino]-3-(phenylthio)propan-2-ol	1	0	
AB_0001290	3-[(4-chlorophenoxy)methyl]-5-[(2-pyridylthio)methyl]-1,2,4-oxadiazole	2	1	0
AB_0001300	3-(2-thienylcarbonyl)-4h-pyrido[1,2-a]pyrimidin-4-one	1	0	

As regards table 1, one compound (Compound AB\_0001010) showed a positive NMR signal even in the absence of target antibody. That compound is likely to be a false positive and was therefore excluded from further analysis. A total of 22 compounds did not show any binding signal in the NMR experiments performed at highest antibody concentration and were thus designated as non-binders. From 23 compounds which showed signal at the highest antibody concentrations a total of 14 did not show any signal at the first dilution of antibody concentration. These compounds were designated as weak binders. Nine compounds showed some kind of signal at the first dilution of antibody concentration and were thus designated as medium to strong binders. Of these, three compounds AB\_0000860, AB\_0000910 and AB\_0001250 showing a clear signal (2 in table 1) were further analysed at a second dilution of antibody concentration (conc 3 in table 1). Whereas compound AB\_0000910 did not show any signal at this concentration, both AB\_0000860 and AB\_0001250 did and were thus confirmed as strong binders.

As regards the structure-activity relationships, the following observations arose from inspection of the structures of the compounds belonging to the three groups of non-binders, weak binders and medium to strong binders. Preferable for binding seems to be the combination of an aromatic part with and aliphatic part with appropriate elements on both parts. Positive for binding for the aro-

matic part is a *meta*- and/or *para*- substituted phenyl ring without heteroatoms in the ring. Especially the presence of nitrogen in the ring seems to influence binding negatively as well as substituents in *ortho* position. Preferable for binding for the aliphatic part are 1) the presence of a tertiary anilinic nitrogen attached to position 1 and 2) (only) one  $\beta$ -keto group attached to position 1, position 1 being the position where the aliphatic part of the ligands is connected to the assumed deepest laying aromatic ring. Preferably a combination of both features like in the N,N-alkylated urea moiety found in the two hits confirmed as strong binders. AB\_0000860 possesses two aromatic rings differing by their relation to the keto groups of the hydantion ring. This asymmetry gives the molecule a direction, which from the docking analysis agrees with the requirement of only one keto group in a  $\beta$ -position relative to position 1.

The presence of two keto groups in a  $\beta$ -position relative to position 1 disfavors binding. This is in agreement with docking results, where it can be seen that a second keto group would probably be forced into a rather unfavourable hydrophobic environment. Also, larger ring-systems than six member rings (for instance fused rings) seem to have a negative influence for binding. Among the weak binders five compounds were found containing non-substituted phenyl rings and three compounds containing tri-fluoro-methyl groups. It could be speculated that for these compounds the affinity detected may be related to hydrophobic interactions of a rather non-specific type.

### Example 2: Directed library

#### Actions undertaken after the analysis of the NMR screening

From the analysis of the results, two directed libraries centred on the structures of the confirmed strongest binder AB\_0001250 were created.

### Directed library centred on the structure of AB\_0001250

Hit AB\_0001250 was one of the hits designated as strong binders. Also, the structure as such offered a potentially attractive synthetic route for varying the substitution pattern of central motif, i.e. the tetra substituted urea, including the introduction of a handle for immobilisation, e.g. to a gel. Therefore, AB\_0001250 was chosen as a starting point in the continued development of improvements.

The analysis started with a search for varying the substitution pattern of the aromatic ring. The di-chloro substituted aromatic ring that is present in AB\_0001250 does according to the docking fill the available space in an appropriate way in two dimensions but, since that structure is planar, a pocket above the plane of the ring was not filled.

3-chloro-4-methoxyaniline was chosen as the starting point for further synthetic work, since it can be converted into the desired starting material by alkylation of the anilinic nitrogen with methyl iodide.

One option is to have a fluoro-substituent in the *meta* position, in order to favour hydrogen bonding of protein residues. Also for this, a suitable starting material, namely 3-chloro-4-methoxyaniline, is commercially available. The compounds belonging to the designed directed library together with the structure of the original hit AB\_0001250 as shown in Figure 3 were subject to docking and NMR screening.

### Example 3: Molecular modelling and docking of directed library

The modelling and docking was performed as described above under Materials and Methods. The results from the docking are as follows:

All docked compounds in the directed library with the exception of AB\_0001256 resulted in a docked conformation inside the binding pocket, which very closely resembles the position of the docked hit AB\_0001250, see Figure 4. In four of the compounds, this is the best-ranked solution. In one of them (AB\_0001252), the solution corresponding to the molecule inside the pocket is the second ranked solution. AB\_0001256 lacks one of the methyl groups in the tetra substituted urea moiety. Consequently the corresponding amide bond should be more constrained to a planar geometry as compared to the remaining compounds in the library for which such geometry is forbidden because of steric effects between the methyl groups. Apparently, the non-planar geometry is of importance for docking.

The values of the obtained expected energies of binding in kJ/mol are -10, -13, -12, -14 and -14 for AB\_000125 (-1) to (-5) respectively. Orthographic plots of the docked hits are shown in Figure 5.



Example 4: NMR screening of directed libraryTable 2: Results from NMR screening of the directed library

Concentration code as follows: conc. 1 means 500 and conc 2 100 nM anti-body. NMR signal code: 0 no, 1 weak and 2 strong signal, nd means not determined.

ID	Trivial name	Conc 1	Conc 2
AB_0001251	4-(1,3-Dimethyl-3-phenyl-ureido)-butyric acid methyl ester	0	0
AB_0001252	4-[3-(3-Fluoro-4-methoxy -phenyl)-1,3-dimethyl-ureido]-butyric acid methyl ester	1	1
AB_0001253	4-[3-(3-Chloro-4-methoxy -phenyl)-1,3-dimethyl-ureido]-butyric acid methyl ester	2	1
AB_0001255	4-[3-(3,4-Dichloro-phenyl)-1,3-dimethyl-ureido]-butyric acid methyl ester	2	2
AB_0001257	4-[3-(3,4-Dichloro-phenyl)-3-methyl-ureido]-butyric acid methyl ester	2	0
AB_0001258	4-[3-(3,4-Difluoro-phenyl)-1,3-dimethyl-ureido]-butyric acid methyl ester	1	1
AB_0003090	4-[3-(3,4-Dichloro-phenyl)-1,3-dimethyl-ureido]-butyric acid		
AB_0003290	1-[(3,4-Dichloro-phenyl)-methyl-carbamoyl]-pyrrolidine-2-carboxylic acid methyl ester (R-isomer)	2	2

The compounds AB\_0001251 through AB\_0001259 and AB\_0003130 through AB\_0003150, shown Table 2, where screened at the two higher antibody concentrations. The result showed that all compounds except AB\_0001251 were interacting with the antibody. It was also shown that compound AB\_0001255, which is the original compound AB\_0001250 with an extension, has the strongest binding of these compounds in the assay. Further, the results also showed that substituents on the aromatic ring are indispensable for binding in

this type of compounds since the only negative result was obtained with the unsubstituted variants AB\_0001251 and AB\_0003150.

Example 5: General method for attaching ligand to support

Sepharose™ HP (Amersham Biosciences, Uppsala, Sweden) that had previously been derivatised with allyl glycidyl ether was activated with bromine and coupled with hexamethylene-diamine according to a standard protocol. The free amine content was determined to 17 µmol/mL gel according to a standard protocol.

2 mL of this gel was transferred to a reaction vessel together with 2 mL of DMF. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.15 mmol) and diisopropyl amine (0.1 mmol) was added and the suspension was put on a shaker at 30 °C. After 5 min. the ligand to be coupled (0.1 mmol) was added and the reaction was allowed to continue for 15 hours.

Thereafter the gel was transferred to a glass filter funnel and washed with a 1:1 mixture of DMF and acetic acid anhydride. The gel was allowed to be in contact with this solution for 30 min. whereafter it was washed with consecutively DMF, water, and 20 % ethanol.

The amount of ligand coupled to the gel was determined with a NMR-method using tri-methoxy benzene as internal reference.

Example 6: Chromatographic characterisation of affinity media containing the ligand AB\_0003291 according to the invention

AB\_0003291 coupled to Sepharose™ HP (ligand concentration 11 µmol/ml gel as determined by MAS-NMR) was packed in 0.5 ml Tricorn™ 5/20 columns (Amersham Biosciences, Uppsala, Sweden) at a flow rate of 1-2 ml/min. Monoclonals ABFab-K1 (FAB/kappa), ABFab-K2 (Fab'2/kappa) and ABFab-

L2 (Fab/lambda) were tested for binding to AB\_0003291-containing gel using protein concentrations of 0.4 or 0.2 mg/ml in PBS, 1 M  $(\text{NH}_4)_2\text{SO}_4$ , pH 7. 100  $\mu\text{g}$  of ABFab-K1 and 50  $\mu\text{g}$  of the other two proteins were injected at a flow rate of 0.25 ml/min (contact time 2 min) using an Äkta<sup>TM</sup> Explorer 10 chromatography system equipped with a UV cell, pH meter, conductivity cell and auto-injector (Amersham Biosciences, Uppsala, Sweden). Protein loading was followed by a wash period of 20 column volumes of loading buffer (PBS, 1 M  $(\text{NH}_4)_2\text{SO}_4$ , pH 7) and a second wash step with 0.01 M of NaOH.

Figures 7, 8 and 9 show the separate injections of the monoclonals ABFab-K1, ABFab-K2 and ABFab-L2, respectively, on the AB\_0003291-containing media. Evidently, the affinity column containing AB\_0003290 coupled to Sepharose<sup>TM</sup> HP is able to bind the monoclonals ABFab-K1 and ABFab-K2, whereas monoclonal ABFab-L2 directly comes off the affinity column and is found in the flow-through. Elution of monoclonals ABFab-K1 and ABFab-K2 bound to the affinity column in PBS, 1 M  $(\text{NH}_4)_2\text{SO}_4$ , pH 7 is possible using different elution conditions such as 50 mM acetate buffer containing 0.14 M NaCl, pH 4 or PBS, pH 7 containing 10% n-propanol (data not shown).